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Original article

Distribution of chloroplast DNA haplotypes of Japanese beech (*Fagus crenata*, Fagaceae) in Hyogo Prefecture

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Abstract

The chloroplast DNA (cpDNA) haplotypes of the Japanese beech (*Fagus crenata*, Fagaceae) found in Hyogo Prefecture were studied to infer the phylogeographic and colonization histories of the species in and around the prefecture. We sequenced two regions in the 58 individuals selected from 13 populations; these regions were the non-coding region between the *trn*L (UAA) 5' exon and *trn*F (GAA) and the *trn*K region (including *mat*K). Next, their cpDNA haplotypes were determined according to the classification of Fujii *et al*. (2002). Three cpDNA haplotypes were determined as belonging to the Japan Sea alliance. The results suggest that all the Japanese beech species found in Hyogo may have descended from the refugia along the Japan Sea side after the glacial period.

Key words: cpDNA, *Fagus crenata*, Hyogo Prefecture, Japanese beech, refugia, *trn*K, *trn*L- *trn*F

Introduction

The Japanese beech (*Fagus crenata* Blume; Fagaceae) is an endemic woody plant, dominant in the belt of typical cool temperate forests, and widely distributed from southern Hokkaido to southern Kyushu (Horikawa 1972, Environment Agency 1988). There are relatively large areas of *F. crenata* forests in northeastern Japan, however, from central Honshu on the Pacific Ocean side to southwestern Japan, the forests are scattered and isolated in small montane areas generally at an elevation of 200 - 1400 m.

Recently, there have been many biogeographical studies on the Japanese beech using various molecular markers such as variations in allozymes, mitochondrial DNA (mtDNA), and chloroplast DNA (cpDNA) (e.g., Koike *et al.* 1998; Tomaru *et al*. 1997, 1998; Fujii *et al*. 2002; Okaura and Harada 2002; Kobashi *et al*. 2006). These studies have revealed the strong geographic structure of the species. For example, Fujii *et al*. (2002) examined the cpDNA variations in 45 populations from among the entire species' range of *F. crenata*, and found 13 distinct cpDNA haplotypes (types A-M) belonging to two major lineages; these two lineages were basically categorized as belonging to either the Japan Sea or the Pacific Ocean sides of the country. Interestingly, many phytosociological

studies have been conducted on the *Fagus* forests in Japan, and most of the forests were detected as belonging to the two major alliances: the Pacific Ocean side (Sasamorpho-Fagion crenatae Miyawaki, Ohba et Murase 1964) or the Japan Sea side (Saso-Fagion crenatae Miyawaki, Ohba et Murase 1964) (Hukushima *et al*. 1995).

In Hyogo Prefecture, *F. crenata* is distributed from the Japan Sea side to the Seto Inland Sea side (Fukuoka *et al.* 2000).

Hukushima *et al*. (1995) reestimated the phytosociological classification of beech forest in Japan, and showed two types beech forest association in Hyogo Prefecture: the beech forests in the southeastern areas of the prefecture belonging to Sapio japonici-Fagetum crenatae, Sasaki 1970 and the beech forests in the northwestern area of the prefecture belong to Lindero umbellatae-Fagetum crenatae, Horikawa et Sasaki 1959. The former is the forest association observed in Kii Peninsula, Shikoku, and Kyushu Islands, and the latter is the forest association observed from the central to the western Japan Sea side of Honshu Island (Hukushima *et al.* 1995). According to Fujii *et al*. (2002), *F. crenata* from Mt. Hyonosen and Mt. Ohginosen exhibited the cpDNA haplotypes B and C, respectively, these belong to the Japan Sea alliance. Haplotype B is one of the major haplotypes distributed along the Japan Sea side, and haplotype C is unique to Mt. Ohginosen. However, only two individuals were studied from each population, and the cpDNA haplotypes of the remaining Japanese beech populations in Hyogo are unknown.

In most angiosperms, cpDNA is inherited maternally; therefore, cpDNA reflects seed flow, and is a good marker for monitoring the colonization processes (MacCauley 1994). For determining the colonization routes of species, it is important to define the location of the contact zone where populations from different refugia meet after a postglacial expansion (Harrison 1993, Kobashi *et al.* 2006). There are three haplotypes B, C, and F in Kinki District, and of these B and C belong to the Japan Sea alliance as mentioned above, while haplotype F belongs to the Pacific Ocean alliance. Hira Mountain, Shiga Prefecture can be considered the contact zone since both haplotypes B and F were found there (Fujii *et al*. 2002). Hyogo Prefecture might also contain the boundary of the expanding distribution of beeches from the refugia at the Japan Sea and Pacific Ocean sides; however, it is indistinct. In order to understand and determine the biogeographical and migration histories of Japanese beech in and around Hyogo Prefecture, which is considered representative of the elements of a cool temperate forest, we determined their cpDNA haplotypes sensu Fujii *et al*. (2002).

Materials and Methods

We sampled one to twelve trees of *Fagus crenata* from 12 populations each in Hyogo Prefecture. The leaves or winter buds from adult trees were used for DNA extraction. Additionally, we used one specimen deposited at HYO (M. Hashimoto 22222, C1-076586) from Mt. Kasagata to extract DNA, since we could not find any living trees in the field. From fresh materials, total genomic DNA was extracted using slightly modified CTAB method (Doyle and Doyle 1987, Takano and Okada 2002), and CTAB method of Kobayashi *et al.* (1998) was used to extract total genomic DNA from the herbarium specimen. In total, 58 individuals from 13 populations were examined in this study (Table 1). Herbarium vouchers are deposited at HYO.

PCR amplification and sequencing

Two cpDNA regions were amplified by polymerase chain reaction (PCR): the non-coding region between the *trn*L (UAA) 5' exon and *trn*F (GAA), and the entire region was amplified using primers "c" and "f" of Taberlet *et al*. (1991), and the *trn*K region was divided and amplified by upper and lower regions, using two sets of primers: to amplify the upper region, we used primer "3914F" of Johnson and Soltis (1994) and "AF120R" of Fujii *et al*. (2002), and we used "AF (Fagus)" of Fujii *et al*. (2002) and "2R" of Johnson and Soltis (1994) to amplify the lower region. To read the entire sequence of the *trn*K region, we used internal primers "AF250F" and "8R (Fagus) " of (Fujii *et al*. 2002), and additionally made two internal primers "*trn*K 1764F" [TCCTTCAGTGGTGCGGAGTC] and "*trn*K 1180R" [GGGTATTCGTACATCTGTCG]. For non-coding region between the *trn*F 5' exon - *trn*L region, primers "d" and "e" of Taberlet *et al*. (1991) were used to read entire region. The PCR reaction mixtures contained 50-100 ng template DNA, 25ul of 2＊Readymix Taq PCR Reaction Mix with $MgCl_2$, 0.4 μM of each of the primer pairs in a total volume of 25 μL. The PCR program started 94℃ for 3min for initial denaturation, followed by 30 cycles of denaturation 94℃ for 1 min, primer annealing at 50℃ for 1 min and extension at 72℃ for 2 min. The extensions were then extended by 7 min at 72℃. Amplified products were purified using Microspin S-300 HR columns (Amersham Biosciences, Buckinghamshire, England,

Table 1. Source of materials examinated for cpDNA analysis. All localities are in Hyogo Prefecture, Japan.

Population Number	Locality	Voucher	No. of plants
1	Mt. Mikawasan, Mikawa, Kami-cho, Mikata-gun, alt. 200-400 m	A. Takano & S. Fuse 061018-5 (C1- 220963)	4
2	Mt. Kuruhidake, Kuruhi, Toyooka City, alt. 550 m	A. Takano 061009-18 (C1-220813)	5
3	Mt. Ohginosen, enroute from Hataganaru Highlands to summit, alt. ca.1200 m	A. Takano & S. Fuse 060830-2 (C1-220586)	6
4	Mt. Sobudake, Muraoka, Kami-cho, Mikata-gun, alt. 1000 m	D. Fujiki s.n. (C1-201602)	4
5	Mt. Hyonosen, Sekinomiya-cho, Yabu City, alt. 1200 m	A. Takano & S. Fuse 061017-31 (C1- 220843)	4
6	Mt. Fujinashi, Ichinomiya-cho, Shisou City, alt. ca.1050 m	K. Kishimoto & K. Nakamura s.n. (C1- 220699)	1
7	Mt. Mimuro, Chigusa-cho, Shisou City, alt. ca.930 m	R. Shimizu, A. Kawakami, K. Kishimoto & K. Nakamura s.n. (C1-220696)	2
8	Mt. Sankyuan, Mizotani, Ichinomiya-cho, Shisou City, alt. ca.890 m	A. Kawakami & Y. Watanabe s.n. (C1-220698)	4
9	Mt. Ushiroyama, Chigusa-cho, Shisou City, alt. 960 m	A. Kawakami & K. Nakamura s.n. (C1- 220697)	4
10	Mt. Kasagata, Kanzaki-cho, Kanzaki-gun, alt. 800 m	M. Hashimoto 22222 (C1-076586)	1
11	Mt. Kayamachi, Kiyosumi, Aogaki-cho, Tanba City, alt. ca.730 m	R. Shimizu & Y. Watanabe s.n. (C1-220700)	2
12	Mt. Myoken, Kurokawa, Kawanishi City, alt. 660 m	A. Takano 061023-1 (C1-221017)	9
13	Rokko mountain range, Nishinomiya City to Nada-ku, Kobe City, alt. 800 - 900 m	A. Takano 061021-1 (C1-221015, 221016)	12

UK). Sequencing was performed using ABI Prism™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). The products were cleaned following manufacturer's protocol, and were run on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). The 42 of 58 samples, however, the purification of the PCR products, sequencing reaction and DNA sequencing were done by Macrogen, Inc. (Seoul, Korea).

Sequence alignment and haplotype determination

Raw sequences were assembled and edited using the Bioedit program ver. 5.0.9 software (Hall 1999). Then complete *trn*L-F intron and *trn*K sequences were compared with those of each haplotype designated by Fujii *et al*. (2002) and were determined its haplotype.

Results and Discussion

Among the 13 haplotypes determined by Fujii *et al*. (2002), three, i.e., haplotype B, C, and D were found in the 13 beech populations of Hyogo Prefecture (Fig. 1). Four populations in the Japan Sea side were of haplotypes B and C; these haplotypes were also observed in Mt. Mikawasan (B: 1, C: 3), Mt. Kuruhidake (B: 3, C: 2), Mt. Sobudake (B: 1, C: 3), and Mt. Hyonosen (B: 2, C: 2). However, in Mt. Ohginosen, only haplotype C was found among the six individuals studied. In the Rokko mountain range, the 11 beech plants were of haplotype B and one was of haplotype D. All the plants studied in the remaining seven populations were of haplotype B.

This study clarifies that the beech populations in Hyogo Prefecture were of the haplotypes of found in the Japan Sea alliance (haplotypes B and C; Fujii *et al*. 2002). Hukushima *et al*. (1995) suggested that the beech forests of Mt. Myoken and the Rokko mountain range belong to the forest associations of the Pacific Ocean side. However, the cpDNA haplotypes of the beech forests of Mt. Myoken and the Rokko mountain range belonged to the alliance of the Japan Sea side. Fujii *et al*. (2002) also found another example of the discrepancy between phylogeographic pattens and the forest associations in the beeches of Chubu District. Such a contradiction may indicate that the haplotype distribution is more strongly affected by

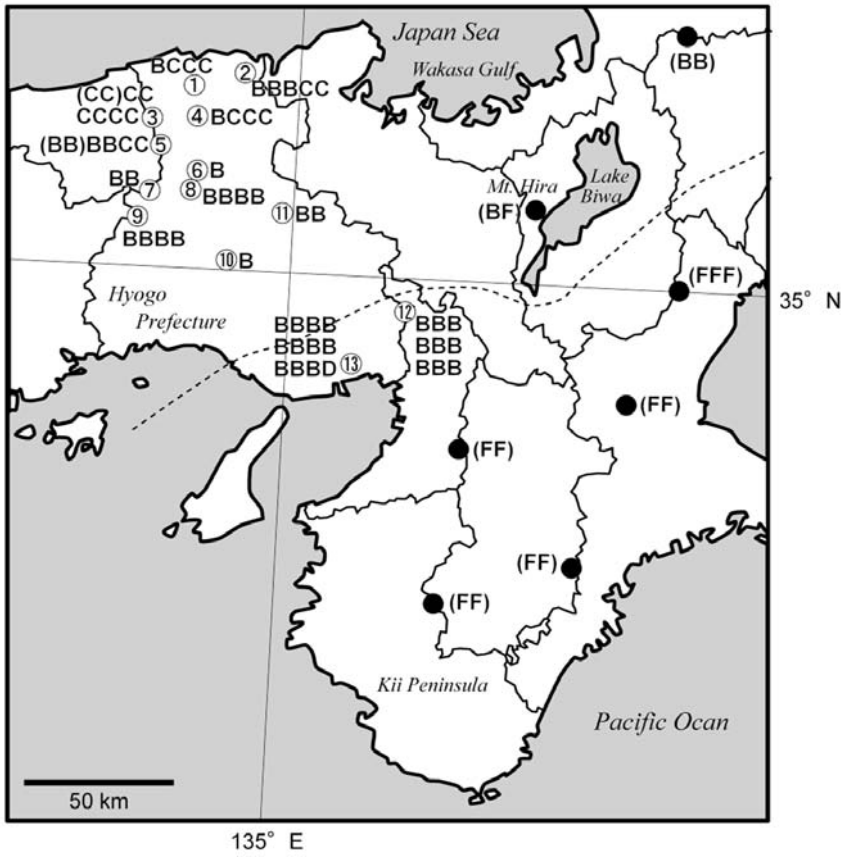

Figure 1. Location of *Fagus crenata* sampling sited for this study. Numbers in circle correspond to the population numbers in Table 1. Letters represent the cpDNA haplotype of the individuals, and those in parenthesis indicate the cpDNA haplotype determined by Fujii *et al*. (2002). Broken line indicates the boarder of Lindero umbellatae - Fagetum crenatae Hirokawa et Sasaki and Sapio japonici - Fagetum crenatae Sasaki (Hukushima *et al*. 1995).

postglacial migration from the refugia of the Japanese *Fagus* than by the current environmental variations. Fossil pollen analysis revealed that the Japanese *Fagus* species migrated southward along the Pacific Ocean and Japan Sea sides of the islands or refugia and extended either northward or to higher altitudes during the interglacial periods (Tsukada 1982a). After the last glacial period (=ca. 12,000 years ago), the *Fagus* forest area began to expand towards the north and interior regions from the coastal refugia (Tsukada 1982b). Considering the results obtained in this study and Tsukada's hypothesis, all the Japanese beech plants in Hyogo Prefecture may expand to the interior regions from the refugia along the Japan Sea after the last glacial period. Therefore, the current forest status of Mt. Myoken and the Rokko mountain range can be explained in two steps: (1) The migration of *Fagus crenata* (and probably some other elements of the beech forest along the Japan Sea side). (2) The invasion or replacement of the beech forest along the Pacific side. Similar genotypic analyses of the different elements of the forest and determining their migration roots will provide an insight into how the current forest association was established.

Haplotype C is found only in the mountains located in the northern part of the Prefecture (i.e., Mt. Ohginosen, Mt. Sobudake, Mt. Mikawasan, Mt. Kuruhidake, and Mt. Hyonosen) (Fig. 1). On the other hand, haplotype B is widely distributed from Tohoku District to Chugoku District (Fujii *et al*. 2002). In inferring colonization routes of haplotypes, it is interesting that only haplotype C was found in Mt. Ohginosen, whereas haplotypes B and C were found in all the other four sites.

The occurrence of haplotype D at Mt. Rokko is rather unexpected. Until now, this haplotype has been known to be found only in Chubu District (Nagano, Aichi, and Gifu Prefectures); therefore its distribution appeared disjunct in this study. It is not to be denied that the individual has haplotype D was planting, because the tree is large and old. The leaves of this individual are obviously larger than the leaves of other individuals in Mt. Rokko. Further studies with a greater number of individuals and populations particularly along the Wakasa gulf or around Lake Biwa would clarify the exact distribution of the haplotype and therefore its putative migration history.

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